

Black tongue in dogs and its relation to sprue, pellagra, and pernicious anemia. The studies of tropical sprue in Porto Rico made it clear that an experimental method would be of great value in studying the nature and treatment of that condition. Moreover, it was thought that animal tests might show a relationship between sprue, pellagra, and pernicious anemia. Several dogs kept on diets low in protein and Vitamin B developed a condition characterized by stomatitis, diarrhea, and central nervous system disturbances in from five to eight weeks. With the exception of the last, the condition resembled strikingly acute tropical sprue. Preliminary observations indicate that disturbances of the gastric function are associated with the other symptoms. It is proposed to test the activity of the gastric juice of these dogs for power to relieve pernicious anemia in human beings. As it has been found possible to prevent the disturbances of the central nervous system, dogs may be kept in a state of chronic illness similar to tropical sprue, and thus it may be ascertained whether this chronic disease leads to experimental pernicious anemia.

(Drs. Avery, Stillman, Goebel, Dubos, Francis, Babers, Goodner, Alloway, and Terrell). The special subjects dealt with in the report are:

The decomposition of the capsular polysaccharide of Type III

pneumococcus by a bacterial enzyme,

Methods of purification and concentration of the specific
enzyme,

Protective action of the enzyme in dermal infection of rabbits with Type III pneumococcus,

Production of pneumococcus pneumonia (Type III) in monkeys.

Isolation of microorganisms decomposing the capsular polysaccharides of other types of pneumococcus.

Chemo-immunological studies on conjugated sugar-proteins.

The specificity of antigens prepared by combining α and β glucosides of glucose with protein.

The synthesis of glucosides of the disaccharides, cellobiose, and maltose.

Studies on the interconvertibility of the specific types of pneumococcus.

The degree and duration of active and passive immunity to pneumococcus in rabbits.

There follows a general account on the immuno-chemistry of the pneumococcus, which is presented in place of the more detailed and technical descriptions contained in the report to the Scientific Directors.

Specific carbohydrates in pneumococcus infection (Oswald T. Avery).

"An important advance in the study of infectious diseases came with the knowledge that bacteria, though simple in form and structure, exhibit differences in biological specificity as sharply defined as are those characteristic of the more complex forms of life. The study of the immunological specificity of microorganisms is not only necessary in the elucidation of the biological relationships existing between varieties of the same species of bacterium, but is essential to the working out of epidemiological problems and to the development of methods useful in the control of infectious diseases by specific therapeutic and prophylactic measures.

"Leaving out of consideration the promising but difficult field of chemotherapy, the problems of specific cure and prevention of infection lie

in the attempt to interpret and imitate by artificial procedures certain protective processes of nature which constitute that which we call immunity. In order to imitate successfully the natural processes involved in spontaneous recovery from disease, it is necessary to know the nature of the specific reactions between the infecting agent and the body tissues of the host. The specificity of these biological processes is advantageously studied by means of the so-called immunity reactions. These serve as a measure of the capacity of the animal organism to produce protective substances, and they afford a means of studying the interaction between these specific antibodies and the infectious microorganism when both are brought together in the animal body or in the test tube.

"Investigations on the specificity of these reactions have added much to our knowledge of the many and diverse problems of infection. The study, however, is one of varying complexity; the methods suitable in one instance fail utterly when applied to another type of infection. While the mechanism of the interaction between host and parasite has to a certain extent been exposed, the immediate problem lies in reconstructing for each microbe a more precise knowledge of the biological properties peculiar to it and the specific reactions which the body develops against it. One approach to this problem is the attempt to relate specific differences in biological behavior to fundamental differences in the function and chemical composition of the component parts of the bacterial cell and to determine the character of the tissue responses to these separate constituents. The total immune response of the host comprises not alone a reaction to the parasite as a whole, but, in addition, the specific and individualized responses to the chemically distinct and immunologically specific bacterial constituents.

"For the past several years we have been seeking to acquire a more intimate knowledge of the relation between the immuno-chemistry and the biological activities of pneumococcus, the most frequent and one of the most fatal of the microbic incitants of pneumonia in man.

"I shall review one phase of the studies, namely, the role of specific carbohydrates in pneumococcus infection and immunity. Renewed impetus was given to the study of pneumonia by the working out of the biological classification of pneumococci which made possible the recognition of sharply defined and specific types within this previously confused species of microorganisms. This has made possible the determination of the frequency of occurrence of specific types of pneumonia, and the recognition of differences in the severity and mortality of the diseases they produce. A study of the presence of pneumococci in the mouth secretions of healthy individuals proved the dissemination of the disease-producing types by healthy carriers and convalescents and suggested a new interpretation of the epidemiology of the disease, while knowledge of type-specificity among pneumococci provided the only rational basis for the possible development of specific treatment by immune serum, which in the case of type I infections, at least, has proved of distinct value.

"Pneumococcus is a unicellular microorganism which is surrounded by an envelope of material known as the cell capsule. This capsular layer is particularly well developed in the case of pneumococci capable of growing and multiplying in the animal body. During growth these encapsulated cells elaborate in the medium of their environment a diffusible substance which in soluble form retains the type-specificity of the bacterial cells from which it is derived. This soluble specific substance is found not only in the filtrates of young cultures, but also in the body fluids of animals experimentally in-

fect, and in the blood and urine of patients during the course of pneumococcus pneumonia. The function of elaborating this specific material is most highly developed in the most virulent organisms. There are grounds for the belief that the capsule of these virulent cells is composed largely of this soluble specific substance. Thus, there is disposed peripherally about the cell an outer layer of capsular substance which reacts specifically with the serum of immune animals. The reaction is remarkably specific, occurring only when the antiserum and the reacting substance are both of the same specific type. These immunological reactions form the basis of the original classification and were worked out before there was any knowledge of the chemical nature of the substances upon which type-specificity depends. The actual isolation of these specific substances in purified form, the determination of their chemical constitution and their relationship to the immunological properties of the cell as a whole are problems to which I shall direct attention.

"The type-specific capsular substances of pneumococcus, first chemically isolated by Dr. Heidelberger, have been found in each instance to belong to the class of sugar-like substances, namely the carbohydrates. No matter from what type of pneumococcus these specific substances are recovered they all possess in common the chemical properties of complex sugars -- the polysaccharides. But, interestingly enough, the capsular polysaccharide derived from each specific type of organism is chemically distinct, each possessing unique chemical properties which serve to differentiate it sharply from the others. Moreover, solutions of these capsular polysaccharides, in chemically purified form, exhibit immunologically the same specificity as do the bacteria of which they originally formed a part. Some idea of how remarkably reactive these sugars are may be judged from the fact that, by the use of an appropriate serum, their presence may be detected in dilutions as

high as 1:5,000,000.

"A study of the chemistry of the capsular polysaccharides has shown that these substances are unusual compounds of simple sugars and uronic acids. Although possessing many properties in common, they exhibit characteristic differences. For example, of the specific substances of the first three types of pneumococcus, the type I polysaccharide differs sharply from the other two in containing nitrogen as an integral part of the molecule, and in possessing basic as well as acidic properties; on the other hand the type II polysaccharide is a dextrorotatory weak acid and the type III a levorotatory strong acid, neither of which contains any nitrogen in the molecule. The fact that the particular constituent determining type-specificity is chemically a carbohydrate is the more striking since immunity reactions have hitherto been considered exclusively the function of proteins. Of equal importance is the fact that this selective specificity is in each instance determined by the chemical constitution of the particular polysaccharide in the capsule and that the presence of this morphological structure conditions both the invasiveness of the parasite and the immune response of the host.

"The fact that polysaccharides elaborated by bacteria growing in the focus of disease may be found in the blood and urine, unchanged in specificity, indicates that the body possesses no enzymes capable of breaking them down into simpler sugars. There is no evidence that these complex bacterial sugars as such are directly responsible for the intoxication accompanying the infection. So far as is known, they are not primarily toxic, at least not in the sense of true bacterial toxins. There are facts, however, which indicate that indirectly at least they may have a harmful effect upon the natural processes of recovery. Because of their specific capacity to bind antibodies, they tend to neutralize the immune substances in the blood and thus

prevent the protective antibodies from reaching the infected areas. Moreover, the capsular polysaccharides are known to exert an inhibiting action on phagocytosis, one of the most important cellular defenses of the body against pneumococcus infection.

"Theoretically, at least, there is no apparent reason why these complex sugars as isolated substances should not by themselves be capable of stimulating the formation of antibodies in the animal body. Assuming that they are of sufficient molecular size, they possess in complexity of structure and colloidal behavior certain properties generally considered essential to the antigenicity of proteins. Indeed, Francis and Tillett have found that in the present state of purity the capsular polysaccharides are capable of inciting antibody production when injected in minute amounts into the skin of convalescents and normal individuals. However, with few exceptions all attempts to evoke any immune response in animals with the highly purified polysaccharides alone have been uniformly unsuccessful. On the other hand, the more these carbohydrates are chemically purified, the more reactive they become in the specific serum of immune animals. Under these conditions it appears that, removed from the bacterial cells, the capsular polysaccharides still retain unimpaired the property of binding with antibodies, although in this form they become quantitatively less active in stimulating antibody production in animals. In this respect they may rightly be included in the group of immunologically important substances which Landsteiner has called haptens, -- substances which have lost more or less completely their antibody-stimulating function without impairment of the property of specifically combining with antibodies.

"The elaboration of the capsular polysaccharide is an important function of the cell. When this function is suppressed or inhibited, as it

may be under certain experimental conditions of growth, the capsule is no longer formed. As a result the organisms lose their type-specificity and exhibit only the common, undifferentiated characters of the species. On the basis of colony differentiation these degraded organisms are spoken of as the "R" or rough forms, and the original encapsulated types are referred to as the smooth or "S" organisms. The unencapsulated R forms of pneumococci, irrespective of their type derivation, are no longer capable of invading the animal body; they have lost their virulence, and are readily taken up and destroyed by the phagocytes of the host; immunologically they exhibit only the species-specificity common to all the degraded R forms of pneumococcus. This transformation resulting in a loss of specific characters may occur in the animal body as well as in the test tube. However, these degraded, avirulent variants do not necessarily remain the harmless saprophytes they were originally thought to be, since it is now known that under suitable conditions they may regain all the specific characteristics that distinguished the original parasitic type from which they came.

"Of even greater biological interest is the phenomenon of the interconvertibility of the specific types of pneumococcus. Griffith of London first showed experimentally by a special technic in mice that R forms derived from one specific type of pneumococcus may be caused to acquire the characteristics of another specific type. This important fact has been confirmed by a number of investigators. In addition, Dawson and Sia by special cultural methods, have found that the actual change from one specific type of pneumococcus to another may be brought about in the test tube outside the animal body.

"The experimental evidence now available seems to indicate that any R strain of pneumococcus has potentially the function of elaborating any one of the specific capsular polysaccharides; -- the particular one being deter-

mined by a particular stimulus of a specific nature. Alloway has recently found that this potential function latent in the living R cells may be specifically activated by the addition to an appropriate medium of a bacterial extract prepared from a given specific type of pneumococcus. Under these conditions, the R forms irrespective of their type derivation again elaborate a capsular material identical in specificity with that of the type of pneumococcus from which the extract was prepared.

"There is at present no certain proof that transformations of this kind ever occur spontaneously in nature. Nor is there as yet any epidemiological or clinical evidence that this form of reversible adaption is a factor in the origin of human infection. However, the experimental evidence leaves no doubt that the non-invasive, non-encapsulated R cells under favorable circumstances are potentially capable of again developing into highly virulent organisms, and that the acquisition of virulence is invariably associated with the restoration of the function of elaborating the specific capsular carbohydrates. Indeed, it is most significant that no matter whether one considers pneumococcus from the viewpoint of virulence, antigenicity, or its capacity to undergo variation, the single determining factor associated with all these characters is the function of synthesizing the specific capsular polysaccharides. Scarcely less important is the fact that the immunological specificity of each of the specific types of pneumococcus depends upon the chemical individuality of the particular carbohydrate in the cell capsule.

"As chemical substances, separate and apart from the bacterial cells, these carbohydrates have been found to incite specific reactions in the tissues of sensitized animals and in the skin of patients convalescent from pneumococcus pneumonia. Guinea pigs passively sensitized with the precipitating serum of an immune rabbit suffer violent anaphylactic shock and die within

three to four minutes following the intravenous injection of as little as .055 mg. of the homologous polysaccharide. The anaphylactic reactions are strictly type-specific. There is now ample evidence to support the view that protein-free, even nitrogen-free, carbohydrates may induce acute anaphylaxis in specifically sensitized animals.

"Tillett and Francis have found that the injection of 0.01 mg. of specific polysaccharide into the skin of patients recovering from pneumococcus pneumonia may evoke an immediate local reaction in the form of a wheal surrounded by a zone of erythema. The cutaneous reactions develop rapidly within fifteen minutes and subside completely in from one to two hours; they are elicited only by the specific polysaccharide derived from the same type of pneumococcus as that causing the infection in the patient. The capacity of the skin to react to the specific bacterial sugar is intimately associated with recovery and closely parallels the occurrence of type-specific antibodies in the patient's serum. The results indicate that this specific skin test has prognostic significance and may become of value in determining the therapeutic dosage of antipneumococcus serum.

"Studies on 'synthetic antigens' prepared by chemically combining derivatives of glucose and galactose with proteins have shown that even these simple sugars exert a determining influence on the immunological specificity of compounds of which they form a part. The newly acquired specificity of these artificially conjugated sugar-proteins is in each instance determined by the chemical structure of the carbohydrate irrespective of the protein to which it is attached. It is especially significant in the case of glucose and galactose that the two sugar derivatives differ from each other only in the spatial arrangement of the hydrogen and hydroxyl groups on a single carbon atom. It is a remarkable fact that the mere rotation of this carbon atom

through an angle of 180° suffices to change completely the antigenic specificity of two substances otherwise chemically identical. In the case of these artificially prepared sugar-proteins, the two isomeric sugar derivatives can be selectively differentiated one from the other by serological methods. These observations on the immuno-chemistry of carbohydrates confirm the original studies of Landsteiner on the specificity of azo-proteins and furnish additional evidence of the general dependence of immunological specificity upon the chemical constitution of the reactive substances. It is evident, therefore, that simple sugars, which by themselves are non-antigenic, may, when coupled to a protein, specifically determine the immune response of treated animals, and that the antibodies thus engendered reflect the orienting influence of the sugar radical on the specificity of the antigen as a whole.

"From these results we were led to test the possibility of 'synthesizing' an artificial bacterial antigen. For this purpose the capsular polysaccharide of type III pneumococcus was chosen, since it contains no nitrogen and in its present state of purity may be regarded as a definite chemical entity. Moreover, if results were obtained with this particular polysaccharide they would be the more significant, since the free substance by itself has never been found to elicit any immune response in rabbits, and even the original bacterial cells from which it is derived fail in a majority of instances to incite specific antibodies in these animals. From a chemical point of view, the difficulty lay in synthesizing the appropriate derivative of this complex sugar. It must be one capable of being coupled to protein and one in which the chemo-specific groups of the polysaccharide are not masked by the chemical procedures. Dr. Goebel succeeded in synthesizing the amino-benzyl-ether of the type III polysaccharide and in coupling the diazonium derivative with a foreign protein, namely, the globulin from horse serum. This soluble antigen

therefore has in common with type III pneumococcus only the specific capsular polysaccharide. Rabbits injected with this artificial antigen uniformly developed in their serum type-specific antibodies. The antiserum thus produced not only precipitates the original polysaccharide, but agglutinates living cultures of type III pneumococcus and protects animals against infection with virulent organisms of the homologous type.

"Knowledge of the chemical nature and significance of the capsular polysaccharides in pneumococcus infection and immunity led us to search for enzymes capable of decomposing these specific carbohydrates. A number of enzymes of animal and plant origin as well as cultures of various bacteria, yeasts, molds and soil actinomycetes, many of which were known to decompose cellulose and other complex carbohydrates, were tested without success. My associate, Dr. Dubos, isolated from peat soil a bacillus which possesses an enzyme that acts specifically on the capsular polysaccharide of type III pneumococcus. From these bacilli the active enzyme has been extracted in soluble form. By technical procedures, active preparations of the enzyme have been purified and concentrated without appreciable loss in potency.

"In view of the marked differences in the chemical composition of the various capsular polysaccharides, it is not surprising to find that the enzyme decomposes only the type III substance and has no effect upon any of the other bacterial sugars thus far tested. In this respect, the selective action of the enzyme is as specific as in the immune reaction between the type III polysaccharide and its homologous antibody. The polysaccharide acted upon by the enzyme loses its serological specificity and is no longer precipitable by type III antipneumococcus serum. This enzyme not only acts on the chemically isolated sugar, but it specifically decomposes this substance in the native form in which it exists in the capsules of the living cells, When a

sterile solution of active enzyme is added to a growing culture of type III pneumococci, the organisms lose their specific agglutinability and the soluble capsular polysaccharide is serologically no longer demonstrable in the culture fluid. Under these conditions, the enzyme decomposes the capsular substance as rapidly as it is formed without impairing the viability of the decapsulated organisms.

"The action of the enzyme does not result in a loss of the function of elaborating the capsular substance, since pneumococci so treated promptly regain their capsules when transferred to an enzyme-free medium. The active enzyme, therefore, represents a specific agent which by itself is neither bactericidal nor bacteriolytic but which, by decomposing the capsular structure, completely alters the biological behavior of the bacterial cell.

"In view of these findings, experiments were carried out to determine whether the enzyme would favorably influence the course of experimental infection in mice with type III pneumococcus. It was found that a single injection of an active preparation of enzyme protected mice against infection with a million times the number of virulent organisms invariably fatal in the untreated animals. The protective action of the enzyme is type-specific; just as in the test tube it decomposes only the type III polysaccharide, so in the animal body it is effective only against infection with type III pneumococcus.

"Experimental evidence indicates that in mice the enzyme also has a curative action when administered in the course of an infection already well established at the time of treatment. The administration of the specific agent as late as eighteen hours after the onset of infection has brought about the recovery of mice infected with multiple lethal amounts of a virulent culture of type III pneumococcus. Experiments carried out in collaboration with Dr. Goodner and Dr. Dubos have shown that the enzyme has also a marked curative

action in the disease brought about by infecting rabbits intradermally with a highly virulent strain of type III pneumococcus. The experimental disease is characterized by the rapid development at the site of inoculation of an intense inflammatory lesion with spreading edema, marked cellular infiltration and hemorrhagic necrosis, accompanied by fever and the early invasion of the blood stream with increasing numbers of pneumococci. The infection ordinarily terminates fatally within three to four days in 95 per cent of untreated rabbits. Following the intravenous injection of an adequate amount of active enzyme, the bacteremia promptly disappears; the local lesion, freed of bacteria, undergoes the natural processes of healing, and recovery occurs in 95 per cent of the animals so treated.

"The experimental results support the view that the primary action of the enzyme lies in its capacity to decompose the capsular polysaccharide of the invading pneumococci. The process of decapsulation brought about by the direct action of the enzyme strips the bacteria of their capsular defense and thereby exposes their naked and unprotected bodies to direct attack by the phagocytes of the host. Thus phagocytosis, ineffective against the encapsulated forms, now becomes the important mechanism in the final destruction of organisms from which the capsular substance has been removed by the action of the enzyme.

"In this sense, the enzyme may be said to initiate a protective reaction, the successful issue of which depends upon the effective phagocytic response of the host. For these reasons, it at once becomes apparent that the curative action of the enzyme is subject to the limitations imposed by the variations that occur in the cellular defense of the infected animal.

"These studies suggest that the capsule -- long recognized as a defense mechanism on the part of virulent bacteria -- is a decisive factor in

determining the fate of pneumococci in the animal body and that this structure is vulnerable to attack by agents other than specific antibodies."

Studies on Hemophilus influenzae (Drs. Cole and Pittman). The study of bacteria of the genus Hemophilus described in the last report has been continued. Strains of all species isolated from the respiratory tract are still being studied; the greater emphasis, however, has been placed on an investigation of the immunological relationship of S and R variants which were derived from these S strains, the production in a horse of a specific antiserum against Type b bacilli, and a method of determining the protective potency of this antiserum.

Type b H. influenzae antiserum has been produced in a horse. Several hospitals have been supplied with the serum, and a few patients suffering with influenzal meningitis have been treated with it. While the results have thus far been unsatisfactory, it has not been used in a sufficient number of cases to form definite conclusions concerning its efficacy. At present strains from 22 cases of influenzal meningitis have been studied, and they have all been of Type b with the exception of one, which was not type-specific and which produced rough (R) colonies.

That the Type b antiserum contains a protective substance has been definitely shown by the prevention of the lesion (injuries) which Type b organisms induce in the skin of rabbits. This protective substance can be quite accurately measured. The smallest amounts of the sera from the three large successive bleedings that would protect against the "standard skin lesion dose" were, respectively, 1/200, 1/600 and 1/800 cc. The "standard skin lesion dose" of culture is ten times the smallest amount of culture which will induce a lesion that remains positive for three days. It is of interest to note that the protective titre of the serum has steadily increased during a

course of immunization of more than a year. The serum is apparently type-specific in its action, since it only prevents the skin lesion induced by Type b organisms. The development of the lesions following the inoculation with other type-specific strains and the R variants is apparently not influenced by the immune serum.

(Drs. Rivers, Berry, Benjamin, Sprunt, and Schwentker). Patients with a variety of infectious diseases have been admitted to the ward for clinical studies, particularly in regard to the changes in the blood picture as revealed by the supravital method of staining, and for the purpose of obtaining material with which animals and tissue cultures were inoculated. The case of agranulocytosis proved to be of interest because it occurred following tonsillectomy and responded favorably to intravenous injections of adenine sulphate.

Cultivation of vaccine virus for use in man. In a previous report a simple medium for the artificial cultivation of vaccine virus was described. The results of the work indicated that vaccine virus is capable of multiplication in the presence of minced chick embryo tissue suspended in Tyrode's solution. The experiments, however, were conducted with a neurovaccine virus, and, although the active agent obtained caused typical vaccinal lesions in rabbits, it was deemed best not to test it in human beings. To obtain a culture virus for Jennerian prophylaxis in man, it seemed advisable to adapt a dermal strain of vaccine virus to our method of cultivation. The report deals with the results of this work.

Vaccine virus, Lot 611, prepared by the New York City Board of Health was used to initiate the cultures. Having rid the dermal vaccine virus of bacteria it remained for us to determine whether it was possible to